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Simplified Methods for Construction, Assessment and Rapid Screening of Peptide Libraries in Bacteriophage

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An efficient strategy has been devised for the construction of diverse peptide libraries in bacteriophage vectors. This strategy was used to generate a library of 4×10^8 random decapeptide inserts in the pIII protein of bacteriophage fd. A novel method for evaluating the genetic diversity of bacteriophage libraries based on colony hybridization with partially degenerate oligonucleotides has been developed. The decapeptide library was affinity-selected with a previously characterized monoclonal antibody specific for the V3 loop of the gp120 protein of HIV-1. Immunological screening, an efficient technique for the rapid identification of putative binding bacteriophage, is described. Hexapeptide sequences similar to those obtained from affinity selection of a hexapeptide bacteriophage library were obtained from the decapeptide library in all five frames. Immunological screening of 20,000 clones from the two libraries after two rounds of affinity selection rapidly identified antibody-binding sequences; 93% and 86% of the sequences obtained from the hexapeptide and decapeptide libraries, respectively, had IC50 valuess ≤ 10 mM as free peptides.

Keywords: bacteriophage; genetic diversity; immunological screening; peptide library

1. Introduction

The construction of diverse recombinant peptide libraries coupled with affinity selection and screening methods provide a powerful tool for the rapid identification of peptide ligands. Filamentous bacteriophage peptide libraries have been used to identify antibody epitopes (Scott & Smith, 1990; Cwirla *et al.*, 1990), and streptavidin-binding peptides (Devlin *et al.*, 1990). Phagemid libraries have been used to identify higher affinity variants of human growth hormone (Lowman *et al.*, 1991) as well as antibody epitopes (Felici *et al.*, 1991). Bacteriophage libraries displaying Fab fragments have been used to present the human (Marks *et al.*, 1991) and murine (Clackson *et al.*, 1991) immunological repertoires. Here, we describe an efficient strategy utilizing self-priming partially degenerate oligonucleotides for the generation of a bacteriophage peptide library of great size and complexity that should be applicable to the construction of any diverse library.

A colony hybridization technique was developed to evaluate the genetic diversity of a decapeptide bacteriophage library. The genetic diversity of bacteriophage libraries has been examined by two different techniques to date. Cwirla *et al.* (1990) examined the diversity of an N-terminal hexapeptide library in pIII by analyzing the distribution of bases at each position within the codons *via* mass sequencing of pooled clones. Marks *et al.* (1991) have estimated the diversity of a V-gene library by PCR§ amplification of library colonies (Güssow & Clackson, 1989) followed by restriction analysis and sequencing. Both of these techniques yield a general qualitative picture of the genetic diversity of a phage library. The modified colony hybridization technique described here more rigorously assesses library diversity.

An immunological colony screening technique was utilized for the rapid screening of affinity-selected peptide libraries. Immunological screening has been used in the screening of cDNA libraries

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§ Abbreviations used: PCR, polymerase chain reaction; bp, base-pair(s); PEG, polyethylene glycol; u.v., ultraviolet light; TMAC, tetramethylammonium chloride; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Ig, immunoglobulin; IC50, concentration required for 50% inhibition of binding; FMOC, 9-fluorenylmethoxycarbonyl; ELISA, enzyme-linked immunosorbent assay; t.u., transducing unit.

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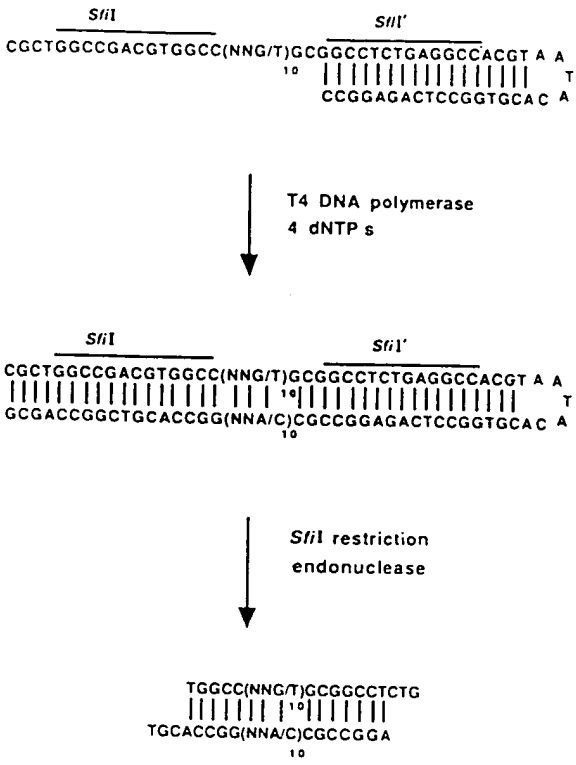

Figure 1. A representation of the strategy utilized to insert library oligonucleotide sequences into the pIII gene of Fuse 5 bacteriophage vector. The self-annealing, partially degenerate oligonucleotide is self-priming and allows the generation of a bacteriophage peptide library of great size and complexity. N in the nucleotide sequence indicates that an equimolar mixture of the 4 nucleotides was utilized in the position during oligonucleotide synthesis.

constructed in plasmids (Broome & Gilbert, 1978) and bacteriophage λ expression vectors (Young & Davis, 1983).

The standard procedure for examining the output of an affinity selection experiment has been the DNA sequencing of random clones (e.g. see Scott & Smith, 1990; Devlin *et al.*, 1990; Cwirla *et al.*, 1990), which is a labor-intensive process. The immunological screening method presented here represents an improvement over the standard procedure by allowing the direct identification of binding bacteriophage.

2. Materials and Methods

(a) *Strains, bacteriophage, DNA sequencing and enzymes*

Escherichia coli strains used were K91(Hfr-C *thi*) (Scott & Smith, 1990) and DH10B (*ara*D139 Δ(*ara, leu*)7697 Δ*lac*X74 *gal*U *gal*K *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *rps*L *deo*R ϕ80d *lac*ZΔM15 *end*A1 *nup*G *rec*A1). Electrocompetent DH10B cells were obtained from Gibco BRL. The bacteriophage vector used for library construction was Fuse5 (a generous gift from G. Smith). Dideoxynucleotide sequencing (Sanger *et al.*, 1977) was performed using the Sequenase 2.0 system (United States Biochemicals). Restriction enzymes and phage T4 DNA ligase were obtained from New England Biolabs (NEB).

(b) *Decapeptide library construction*

The strategy utilized to insert random peptide sequences into the amino terminus of pIII for the decapeptide library is illustrated in Fig. 1. To obtain double-stranded oligonucleotides containing a degenerate sequence flanked by 2 *Sfi*I sites compatible with those of the Fuse5 vector, an 88 base library oligonucleotide was synthesized with a self-complementary 3′ terminus, which allows formation of a self-priming hairpin structure (Maniatis *et al.*, 1976). The complementary strand was synthesized by extension of the 3′ end with T4 DNA polymerase. The *Sfi*I compatible ends were generated by restriction enzyme digestion and the desired fragment electrophoresed on a 15% (w/v) polyacrylamide gel. The DNA of the correct size was then excised, electrophoresed into a 4% (w/v) NuSieve agarose gel (FMC) and the DNA recovered from the agarose gel using the Mermaid oligonucleotide purification kit (BIO 101).

After digestion of the Fuse5 phage vector with *Sfi*I, the 14 bp spacer sequence was removed by precipitation with isopropanol (Sambrook *et al.*, 1989). A total of 20 μg (3·4 pmol) of vector was then ligated with 200 ng (6·8 pmol) of purified oligonucleotide insert overnight at 15°C in a volume of 4 ml with GIBCO BRL ligase buffer and 4000 units of T4 DNA ligase (NEB). The ligation mix was extracted twice with an equal volume of phenol/chloroform (1:1, v/v), once with an equal volume of buffer-saturated chloroform and then precipitated with ethanol, washed twice with 70% (v/v) ethanol and dried. The ligation mix was resuspended in 40 μl of water. Transformation of 4 μl of ligation mix into 80 μl of electrocompetent DH10B cells (GibcoBRL) was performed by electroporation using a Gene Pulser electroporation apparatus (Biorad) at 1·8 kV/cm, 200 ohms, 25 μF in 0·1 cm cuvettes (Dower *et al.*, 1988) for a total of 10 transformation mixes.

Each of the transformation mixes was immediately resuspended into 2 ml of 2 × YT medium (yeast/Bactotryptone medium; Miller, 1972) containing 0·2 μg tetracycline/ml and allowed to recover and express the tetracycline resistance gene, at 37°C for 1 h with 225 revs/min agitation. Portions (20 μl) of cells from each of the transformations were removed and various dilutions plated on LB (Luria-Bertani medium; Miller, 1972) plates containing 20 μg tetracycline/ml to determine the number of independent transformants and to check electroporation efficiency. The 10 transformation mixes were separated into 5 pairs and each pair was diluted into 800 ml of 2 × YT medium (with 20 μg tetracycline/ml) and allowed to grow for 18 h at 37°C at 325 revs/min to $A_{600} = 2{\cdot}0$. The phage supernatant was then cleared of cells and debris by centrifugation at 8000 revs/min (6800 *g*) for 10 min at 4°C in a Sorvall GS3 rotor. The supernatant was precipitated twice with polyethylene glycol 8000 (PEG) overnight at 4°C at a concentration of 3·3% (w/v) PEG, 0·4 M-NaCl. The PEG precipitate was resuspended in a final volume of 7·5 ml in TBS buffer (50 mM-Tris·HCl (pH 7·5), 150 mM-NaCl).

(c) *Evaluation of the genetic diversity of the decapeptide library*

The genetic diversity of the decapeptide library was evaluated using a colony hybridization protocol in which probe dissociation is independent of sequence (Wood *et al.*, 1985). Four ^{32}P-labeled 17 bp oligonucleotide probes (see Fig. 2) that hybridize in part to the degenerate region of the insert (6 bases) and in part to the constant region of the insert (11 bases) were used to probe the same 5000

clones from the decapeptide library. K91 infected with library phage were plated on a total of 10 plates (150 mm × 15 mm) of LB medium containing 20 μg tetracycline/ml. The colonies were transferred and cross-linked to nylon membranes under u.v. light (Stratagene Duralon-UV). Treatment of the membranes and u.v. cross-linking were performed as described by the manufacturer (Stratagene, UV Stratalinker 1800; 1200 μJ, 30 s). The membranes were pre-hybridized at 42°C in 5 × SSC (750 mM-NaCl, 75 mM-trisodium citrate, 40 mM-sodium phosphate (pH 7·5), hybridization buffer (5 × Denhardt's solution; albumin/polyvinylpyrrolidone/Ficoll each at 1 mg/ml diluted in 5 × SSC containing 100 μg denatured salmon sperm DNA/ml). Oligonucleotide probes were phosphorylated as described by Sambrook *et al.* (1989). Following pre-hybridization, labeled probe ($2·0 \times 10^6$ cts/min per ml) was added to the pre-hybridization solution and the hybridization allowed to proceed overnight at 42°C with gentle agitation. The filters were washed 3 times in 0·1% (w/v) SDS, 2 × SSC at 42°C with gentle agitation (10 min wash). The filters were rinsed briefly in water and then in washing buffer: 3 M-tetramethylammonium chloride (TMAC), 50 mM-Tris·HCl (pH 8·0). The filters were washed in a shaking water-bath in washing buffer (1 h washes). The washing temperature that selected for perfect hybrids was determined empirically by use of a set of control filters. Each control set consisted of filter-lifts of colonies containing control phage with appropriate inserts: one insert was a perfect match to the oligonucleotide probe, and the other differed in sequence by a single base from a perfect match to the oligonucleotide probe. After washing, the filters were placed under film (Kodak XAR-2) for 4 h. After analysis of the control filters, the washing step was repeated, and the washing temperature increased sequentially in 2 deg.C increments from 50°C, until a temperature was reached at which all but perfect matches were eliminated, as revealed by the control filters. Washing temperatures approaching 56°C selected for perfect hybrids for all 4 probes, as was predicted by Wood *et al.* (1985). Positive clones from the decapeptide library that hybridized to the probes were confirmed as perfect matches by DNA sequencing.

(d) *Affinity selection*

The decapeptide library was subjected to a modification of the affinity selection technique of Parmley & Smith (1988). For comparison, a hexapeptide library donated by G. Smith was affinity-selected. The monoclonal antibody used for affinity selection was generated by immunization with recombinant Env 2-3 of human immunodeficiency virus (SF-2 isolate of HIV-1; Chapman *et al.*, 1991). The monoclonal antibody (mAb) was mapped to a 10 residue epitope (RAFHTTGRII) on the v3 loop of gp120 (Haigwood *et al.*, 1990). The antibody was biotinylated with biotin-XX-NHS (Calbiochem) as described by Parmley & Smith (1988).

Two rounds of affinity selection were performed. Streptavidin-coated, oxirane-derivatized polyacrylamide beads (Bass *et al.*, 1990) were used for the first round of affinity selection to allow an excess of streptavidin sites (and therefore the number of antibody sites) over the number of phage particles. The beads (Sigma, 1 μm particle size) were coated with streptavidin by incubating 500 mg of beads with 1 mg streptavidin/ml in 1 ml of phosphate-buffered saline (PBS) for 48 h at room temperature with gentle agitation. The beads were blocked with a solution of 1 mg bovine serum albumin/ml for 24 h at 4°C, followed by 10 ml of 5% (v/v) β-mercaptoethanol in PBS for 24 h at 4°C with gentle agitation. The beads were then washed 5 times with 10 ml of PBS and stored in PBS containing 1 mg bovine serum albumin/ml.

The first round of affinity selection was performed by incubating 50 μl of biotinylated IgG (7·5 μg) with the streptavidin-coated beads (10 mg, 10 nmol/g resin) for 1 h at room temperature with agitation (a final antibody concentration of 1·0 μM). The beads were washed with PBS, 0·1% albumin. The library phage (10^{10} to 10^{11} transducing units in 50 μl) were then added to the antibody complexed beads and incubated for 2 h at room temperature with gentle agitation. The beads were washed by alternately suspending and pelleting 7 times with 400 μl of PBS, 0·5% (v/v) Tween 20. The bound phage were eluted with 0·4 ml of 0·1 M-glycine (pH 2·2), 1 mg albumin/ml. After elution, the pH of the phage suspension was adjusted to pH 7·0 with approx. 60 μl of 1 M-Tris·HCl (pH 9·0). The eluted phage were amplified after the first round of affinity selection, as described by Parmley & Smith (1988).

The second round of selection was done as described by Parmley & Smith (1988). This second round was performed under more stringent conditions, using fewer streptavidin sites. Polystyrene Petri dishes (60 mm × 15 mm) were coated with streptavidin (2·5 ml, 3 μg/ml in 0·1 M-$NaHCO_3$, pH 8·6) overnight at 4°C. The plates were then blocked with PBS, 1% albumin for 1 h at room temperature and washed with PBS (3 × 8 ml) just prior to use. The amplified phage library obtained from the first round of affinity selection (48 μl, 10^9 to 10^{10} transducing units) and biotinylated antibody (20 μl, 750 ng, 250 nM) were incubated together for 2 h at room temperature (a final antibody concentration of 10 nM). The antibody/phage mixture was then diluted with 0·5 ml of PBS and added to the streptavidin-coated plates. The solution was incubated for 30 min at room temperature with gentle agitation. The plates were washed 8 times at 5 min intervals with PBS/0·5% Tween 20 (7 ml/wash). The bound phage were eluted and the solution neutralized as above.

(e) *Immunological screening*

The affinity-selected hexapeptide and decapeptide libraries were screened by an immunological colony screening technique adapted from Sambrook *et al.* (1989). Clones from each round of affinity selection were plated on 15 mm × 150 mm LB medium with 20 μl tetracycline/ml plates, at a density of 500 to 1000 well-separated colonies/plate, and were transferred to nitrocellulose membrane filters. The filters were immediately washed twice in TNT buffer (10 mM-Tris·HCl (pH 8·0), 150 mM-NaCl, 0·05% Tween 20, with no lysis step (30 min wash). The filters were blocked with blocking buffer (20% normal goat serum diluted in TNT buffer) for 30 min at room temperature with gentle agitation on a rotary platform. The anti-gp120 antibody was diluted 1:1000 (v/v) in blocking buffer to a final volume of 15 ml/filter and incubated with the filters for 2 h at room temperature with gentle agitation. The filters were then washed sequentially in washing buffer A (TNT buffer containing 0·1% albumin), washing buffer B (TNT buffer containing 0·1% albumin, 0·1% Nonidet P-40) and washing buffer A for 10 min at room temperature with gentle agitation. The secondary antibody, goat anti-mouse IgG–horseradish peroxidase conjugate (Boehringer-Mannheim), was diluted 1:200 (v/v) in blocking buffer to a final volume of 15 ml/filter and incubated with the filters for 2 h at room temperature

Table 1
Control bacteriophage for binding anti-gp120

	Insert sequence†	IC50‡
T bacteriophage	RAFHTTGRH	6·3 nM
I bacteriophage	RAFHTTGAH	320 nM
L bacteriophage	FHTTGRH	3·9 μM

† Amino acid sequence of the insertions present in the pIII protein of the control bacteriophage.

‡ The IC50s were measured by competition ELISA of the free peptide insert, as described in the text.

with gentle agitation. The filters were then washed as above. Positive clones were located by color development using 3,3'-diaminobenzidine tetrahydrochloride, as described by the manufacturer (Pierce). Prior to sequencing, positive clones were verified by a second round of immunological screening. Three control bacteriophage that bind to the anti-gp120 monoclonal antibody with a range of IC50s between 4 μM and 6 nM were screened simultaneously with the libraries as positive controls (Table 1).

(f) *Analysis of candidate peptide ligands*

Candidate peptides (*N*-acetylated, *C*-amidated) were synthesized on an automated synthesizer using FMOC chemistry (Zuckermann *et al.*, 1992). The peptides were assayed in a competition ELISA format over a concentration range of 0·1 nM to 500 μM. Immulon 1 microtiter plates (Dynatech Laboratories) were coated overnight at 4 °C with recombinant gp120 antigen at a concentration of 0·2 μg/well in 50 mM-sodium borate (pH 9·0). Then 50 μl of each peptide was added to the wells, followed by the addition of 50 μl of diluted mAb (26 μM-stock diluted 1 : 50,000 (v/v). The mixtures were incubated in 0·5 M-NaCl, 1% (v/v) Triton X-100, 0·1% (w/v) casein, 250 mM-sodium phosphate (pH 7·5) for 1 h at 37 °C. The plates were washed 6 times with washing buffer (150 mM-NaCl, 0·5% Triton X-100) and incubated with 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1 mg/ml stock diluted 1 : 1000 (v/v). Boehringer-Mannheim) for 1 h at 37 °C. The plates were washed as above and the bound conjugated antibody was quantified by color development with 100 μl of *o*-phenylenediamine (5 mg/ml in 50 mM-sodium citrate, 0·02% (v/v) H_2O_2, pH 5·1). Plates were read at 450 nm on a Molecular Devices ThermoMax microplate reader.

3. Results

(a) *Construction of a bacteriophage decapeptide library*

The decapeptide library was constructed using a self-priming oligonucleotide, which increases the efficiency of both the annealing and priming reactions. Transformation of the library into DH10B cells was accomplished by electroporation. Electroporation of the decapeptide library yielded 4×10^8 independent transformants, at an efficiency of about 2×10^7 transformants/μg. The fraction of clones containing a library insert was determined by comparing the ligation frequency to the religation frequency. The frequency of vector religation was 10^2-fold lower than the ligation frequency. Following amplification, the library was found to contain about 6×10^{12} transducing units (t.u.), or approximately $1{\cdot}5 \times 10^4$ copies of each possible bacteriophage.

5'GGCCGACGTGGCC(NNG/T)$_{10}$GCGGCCTCTGGGGCC 3'

Probe 3'A — AATCGTGCGGCCTCTGG 3'

Probe 3'B — IATCGTGCGGCCTCTGG 3'

Probe 5'A — CCGACGTGGCCTATCGG 3'

Probe 5'B — CCGACGTGGCCTATCGI 3'

Figure 2. The DNA sequences of the 4 oligonucleotide (17 bp) probes used to evaluate the diversity of the decapeptide library. Each of the probes contains 11 bp complementary to either the 5' or 3' constant, flanking sequence of the insert and 6 bp (NGG/T NNG/T; bold face) complementary to the corresponding 5' or 3' end of the 30 bp variable library sequence. Probes 5'A and 5'B differ by a single base-pair of the variable library region, as do probes 3'A and 3'B (underlined).

(b) *Genetic diversity of the decapeptide library*

In order to evaluate the genetic diversity of the decapeptide library, a modified colony hybridization protocol utilizing tetramethylammonium chloride (TMAC) was developed that allows the discrimination of full-length and partial hybrids (i.e. perfect *versus* imperfect matches).

The sequences of the four 17 bp oligonucleotide probes used to evaluate the diversity of the decapeptide library are shown in Figure 2. Each of the probes contains 11 bp complementary to either the 5' or 3' constant, flanking sequence of the insert and 6 bp (NNG/T NNG/T) complementary to the corresponding 5' or 3' end of the 30 bp variable library sequence. Probes 5'A and 5'B differ by a single base-pair in the variable library region, as do probes 3'A and 3'B. Based on the frequency of each possible base-pair at each of the six positions of the sequence (NNG/T NNG/T) of the degenerate region of the probe, the expected number of perfect matches out of the 5000 colonies from the decapeptide library for each of the four probes 3'A, 3'B, 5'A and 5'B is approximately five. The observed number of positives for each probe roughly correlated with the theorized number: 5'A, four positive clones; 5'B, three positive clones; 3'A, three positive clones; and 3'B, four positive clones. As expected, the four probes identified discrete positive clones from the same filter lifts. The three clones that hybridized to the 3'A probe were confirmed as perfect matches by DNA sequencing.

(c) *Affinity selection*

The yield of bacteriophage, defined as the number of bacteriophage obtained in an elution fraction relative to the initial number of bacteriophage, was determined for each round of affinity selection performed on the hexapeptide and decapeptide libraries (Table 2). A significant increase (1000-fold)

Table 2

The yield of phage following two rounds of affinity selection of the hexapeptide and decapeptide libraries

Library	Selection round	Phage yield (%)
Hexapeptide	1	$1{\cdot}2 \times 10^{-4}$
	2	$2{\cdot}2 \times 10^{-1}$
Decapeptide	1	$3{\cdot}7 \times 10^{-4}$
	2	$9{\cdot}5 \times 10^{-1}$

in the yield of bacteriophage from the first round of affinity screening *versus* the second round was observed for both libraries (Table 2). This increase in yield suggests an enrichment of mAb-binding ligands. Random and immunological screenings were performed on the eluted bacteriophage to further confirm this enrichment.

(d) *Random screening* versus *immunological screening*

An increase in the percentage of positive clones between the first and second rounds of selection of both the hexapeptide and decapeptide libraries was observed using immunological screening, as expected from successful affinity selection (see Table 5).

Clones from the second round of affinity selection of the hexapeptide library were randomly chosen for sequencing. Of the 120 clones sequenced, there were 50 distinct peptide sequences (data not shown). Twelve of the peptide sequences were represented more than once. Two of the 12 peptide sequences were represented by clones with differing nucleotide sequences, strongly suggesting that they are not sibling clones. These 12 sequences and eight unique sequences (Fig. 3) were synthesized as 14mer peptides, each containing the hexapeptide library insert sequence and the eight flanking amino acids from the bacteriophage pIII protein

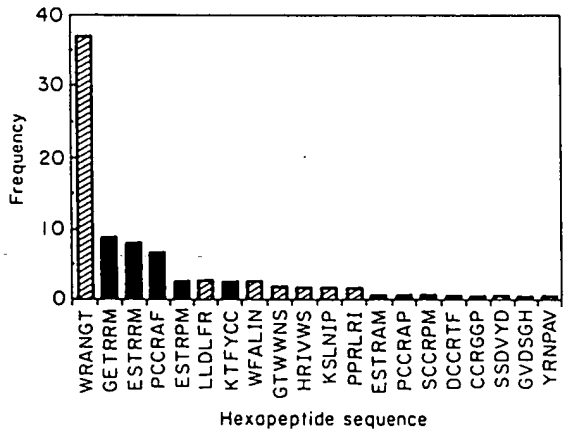

Figure 3. Twenty of the 50 hexapeptide sequences obtained from the random screening of 120 clones, grouped by the frequency with which each appeared. Twelve separate peptide sequences are represented more than once. The 20 peptides were synthesized and assayed for binding with anti-gp120 mAb. Of the 20 free peptides, 10 have an IC50 of > 10 μM (hatched box) and 10 have an IC50 < 10 μM (filled box).

Table 3

IC50s of hexapeptide library epitopes for binding anti-gp120

Insert sequence†	IC50‡	Frequency per 120 clones
ESTRPM	500 nM	3
PCCRAF	630 nM	7
ESTRAM	1·3 μM	1
ESTRRM	1·6 μM	8§
KTFYCC	2·5 μM	3
SCCRPM	2·5 μM	1
CCRGGP	2·5 μM	1
PCCRAP	4·0 μM	1
GETRRM	5·0 μM	9§
DCCRTF	15 μM	1

† Amino acid sequence of the hexapeptide insertions present in the pIII protein of the library bacteriophage.

‡ The IC50s of the free peptides were measured by competition ELISA, as described in the text.

§ The DNA sequences of these isolates differ.

(ADGA[X]$_6$GAAG). The IC50s of the peptides were measured by competition ELISA. Ten of the 20 peptides competed with antigen for binding to anti-gp120 mAb with IC50s of less than 10 μM (Table 3). Two common subsequence motifs were observed: XSTRXM and XCCRX. These motifs appeared in 29% of the 120 clones.

Immunological screening was performed on the hexapeptide library after the second round of affinity selection. Of the 27 immunopositive clones that were chosen for sequencing, 25 contained sequences identical with the submicromolar (IC50) binders identified by random screening (Table 4). Therefore,

Table 4

IC50s of library epitopes chosen by immunoscreening for binding anti-gp120 mAb

Library	Insert sequence†	IC50‡	Frequency
Hexapeptide	**ESTRPM**	500 nM	1
	PCCRAF	630 nM	16
	ESTRRM	1·6 μM	7
	SCCRPM	2·5 μM	1
	VSTRPM	N/A	1
	GSTRRM	N/A	1
Decapeptide	**ESTRPM**AGDG	300 nM	1
	RALE**STRAM**E	100 nM	1
	PSTRPMQRSG	> 10 μM	1
	EIRA**STRPM**R	500 nM	1
	QRSLCCREFP	100 nM	1
	FCCRMPSGGA	1 μM	1
	ECCRPMPHRH	1 μM	1
	SSRPMRLTKT	300 nM	1
	PCCKDTLSRF	3 μM	1
	STGPMRPLQV	600 nM	1
	SSDRAWWCCS	300 nM	1
	GRAWGVARDR	1 μM	1
	LARKDGFGGW	300 nM	1
	SIVSVREVFR	> 10 μM	1

† Amino acid sequence of the hexamer and decamer insertions present in the pIII protein of the library bacteriophage selected by *in situ* immunoscreening. Bold face sequences are either XSTRXM or XCCRX, or are the sequences of known binders.

‡ The IC50s of the free peptides were measured by competition ELISA, as described in the text. N/A, not assayed.

Table 5
Immunopositive clones per round of affinity selection of libraries

Library	Round	Frequency (%)
Hexapeptide	1	2·6
	2	17
Decapeptide	1 pre-amplification	7·2
	1	8·8
	2 pre-amplification	22
	2	25

at least 93% of the clones selected by immunological screening of the hexapeptide library contained peptides with IC50s less than 10 μM, and all either had the XCCRX or XSTRXM motifs as compared with random screening, which yielded a low proportion of binding peptides, 29%. This lower rate could have been predicted by immunological screening, which indicated a 17% frequency of immunopositive clones (Table 5).

Similar results were obtained from the immunological screening performed on the decapeptide library (Table 5). Four of the 14 immunopositive clones selected for sequencing had the XSTRXM motif, and three had the XCCRX motif (Table 4). The remainder of the clones contained unique novel sequences. All 14 sequences were synthesized as free decapeptides and the IC50s of the peptides were measured by competition ELISA.

In order to determine the limit of detection of immunological screening protocol, the three positive control bacteriophage (T, I and L, Table 1) were screened. Only the submicromolar (IC50) bacteriophage-infected colonies (T and I) were detectable with the protocol described above. Therefore, the limit of detectability for the immunological screening technique with the anti-gp120 antibody is an IC50 of approximately 4 μM. Only the T bacteriophage-infected colonies were detected when the concentration of the anti-gp120 antibody was decreased tenfold (1:10,000 (v/v) dilution). Therefore, it should be possible to differentiate between binders with IC50s of 1 μM and 10 nM, by appropriately lowering the concentration of the primary antibody used in the immunological screening.

4. Discussion

We have developed a strategy for generating peptide libraries of great size and complexity utilizing a self complementary, self-priming oligonucleotide. The simplicity of this technique permits the rapid, efficient construction of phage libraries. The decapeptide library constructed using this strategy was large enough to ensure that a complete set of hexamer peptide sequences were represented in the five possible hexamer frames within the decapeptide sequence (a phage library of 4×10^8 with 5 hexamer frames encompasses greater than 1×10^9 hexamers, or $(32)^6$), allowing comparison of affinity-selected peptide sequences from a complete hexamer library *versus* the smaller hexapeptide library (donated by G. Smith). Half of the affinity-selected immunopositive decapeptide library phage chosen for sequencing contained the same two hexamer motifs found in the hexapeptide library, indicating that the anti-gp120 antibody recognized the linear hexamer sequence in the decapeptide library context. In addition, free hexapeptides containing these sequences had equivalent IC50s to those of the free decapeptides (data not shown), indicating that the flanking residues have no effect on the binding of these hexamers.

Neither of the hexamer motifs XSTRXM nor XCCRX is related to the sequence of the original gp120 epitope sequence RAFHTTGRII, and neither these peptide ligands nor the unrelated peptide ligands selected by immunological screening (Table 4) had a equivalent or higher IC50 than the original epitope. It is possible that we are reproducing the same phenomenon seen by others (Scott & Smith, 1990; Cwirla *et al.*, 1990); namely, that peptides of high affinity (nM IC50s) have no selective advantage over peptides of moderate affinity (μM IC50s) under the conditions used to date. It is probable that the epitope RAFHTTGRII was not discovered because the decapeptide library contains only 4×10^{-5}% of a complete decapeptide library.

Like others, we have identified epitopes unrelated in sequence to the known epitope of a monoclonal antibody. Scott & Smith (1990) isolated the epitope CRFVWC by affinity selection with the monoclonal antibody M33, although it bears no sequence similarity to the eliciting myohemerythrin epitope DFLEKI. It can therefore be conjectured that an unrelated combination of residues might approximate the binding valencies and spatial orientation of the residues particular to an eliciting epitope. That unrelated peptide sequences can be affinity-selected by a monoclonal antibody would also imply that the use of peptide libraries for epitope mapping might not be as unequivocal as previously thought. However, it also suggests that phage peptide libraries can be used to great advantage in other applications, such as to investigate cross-reactivities of antibodies.

The presence of a genetic bias in a library decreases the effective size of the library, thereby decreasing the probability that a specific clone will be isolated during screening. Genetic bias can be introduced at many steps during library construction; for example, during synthesis of the variable positions within the library insert, during cloning or as a result of biological selection during library amplification. It is therefore preferable to examine the genetic diversity of a bacteriophage library following its final amplification.

The genetic diversity of the decapeptide library was analyzed using a modified colony hybridization technique (Wood *et al.*, 1985). TMAC mediates a shift of the dissociation temperature (t_d) of A·T base-pairs to that of G·C base-pairs, with the result that the binding of an oligonucleotide probe becomes solely dependent on the length of the

hybrid. Therefore the use of TMAC allows the discrimination of full-length and partial hybrids (i.e., perfect *versus* imperfect matches).

Two sets of oligonucleotide probes were used to examine the genetic diversity of the decapeptide library. These sets were good controls for identifying perfect matches, as the sequences differed only at the final base. Mismatches at the ends of hybrids have less effect on hybrid stability than internal mismatches, therefore, the ability of this method to select for perfect matches was demonstrated unequivocally.

Of the two sets of probes, one set was designed to scan the 5′ and the other the 3′ termini of the variable region in the library insert. Oligonucleotides are synthesized in a 3′ to 5′ direction. Any bias in the sequence of the degenerate region of the library insert due to non-random nucleotide incorporation would be most obvious at the 5′ end of the oligonucleotide, as the effect of an inefficient coupling reaction would be amplified. The analysis of diversity at the 3′ terminus, which is unlikely to show bias, served as a control for the measurements of bias at the 5′ terminus.

If bias introduced during oligonucleotide synthesis was due to the cumulative effect of the inefficient coupling of a single base at every coupling of that base, this bias would be most easily observed if the probes were multimers of single bases (for example, a poly(dG) probe). For reasons of convenience, the probes used in examining the diversity of the decapeptide library were not multimers of single nucleotides. Therefore, the analysis of bias of the decapeptide library was of only moderate stringency. However, this technique can yield a quantitative analysis of the genetic bias of a library if monobase multimers are used as probes.

Affinity selection enriches for the peptide ligands present in a pool of random peptides but it does not eliminate all non-specific peptides, such as the peptides that bind to streptavidin (Devlin *et al.*, 1990). Of the 120 clones selected by the random screening of the hexapeptide library 30% contained the hexamer sequence WRANGT (Fig. 3). Although affinity selection enriched for this hexamer, the free decapeptide containing this sequence did not compete with antigen for binding to anti-gp120 mAb. Interestingly, WRANGT was not isolated during the immunological screening of either the hexapeptide of decapeptide libraries, indicating the effectiveness of the immunological screening technique at identifying binding-site associated phage.

The standard method for screening the output of an affinity selection experiment is the sequencing of random clones followed by analysis of the peptide sequences for motifs prior to further investigation. Felici *et al.* (1991) have described the limited (48 clones) confirmatory immunological testing of affinity-selected bacteriophage *via* dot blot. However, a more efficient approach is to rapidly screen large quantities of clones ($>10^4$) after affinity selection by using a colony immunological screening technique that highlights binding clones. A smaller number of clones therefore needs to be sequenced, since these candidates are likely to bind with a micromolar IC50. At least 93% of the clones isolated by immunological screening of the hexapeptide library contained peptide sequences that bind with IC50s $< 10\ \mu$M. In comparison, random screening yielded a low percentage of binding peptides, 29%. Of the 14 immunopositive clones selected from the decapeptide library, 86% of the identified peptides competed with antigen for binding to anti-gp120 mAb with IC50s $< 10\ \mu$M.

In conclusion, we have generated a peptide library of great size and complexity using a simple and efficient self-priming oligonucleotide scheme. We have examined its genetic diversity by using a modified colony hybridization protocol and subjected it to affinity selection with an anti-gp120 monoclonal antibody. The immunological screening technique described in this publication represents an improvement in bacteriophage library screening as it allows the rapid, direct identification of binding bacteriophage. The immunological screening of affinity-selected libraries is therefore a powerful tool for choosing appropriate peptide ligands as candidates for further investigation.

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